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TITLE: AUTORADIOGRAPHIC DISTRIBUTION AND APPLIED PHARMACOLOGICAL CHARACTERISTICS OF DEXTROMETHORPHAN AND RELATED ANTITISSUE/ANTICONVULSANT DRUGS AND NOVEL ANALOGS

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A study has been carried out in the rat middle cerebral artery occlusion model with dextromethorphan, carbetapentane and three of the carbetapentane analogues, 11, B and D, which were previously examined in the [^{3}H]dextromethorphan and [^{3}H]TCP binding assays (see June 1992 - January 1993 section.)

Method:

Briefly, rats were anaesthetised with Sagatal (60mg/kg) and the carotid arteries exposed. The right artery was tied off and the left clipped for 60 minutes. The brain was exposed using a dental drill and the right MCA occluded by passing an electrical current along a predetermined length. The wound was then closed up, and the animal placed in an incubator until recovered from anaesthesia. Body temperature was monitored throughout the experiment and maintained between 37.5 and 38.5^{0}C.
All drugs were dissolved in saline and administered subcutaneously 30 minutes before occlusion, and also 24 and 48 hours post-occlusion. Vehicle (saline) was administered to a separate group of rats as control. Vehicle and each compound were also administered to sham-operated rats, where the same procedure was followed except that the MCA was not occluded. The rats were sacrificed 72 hours post occlusion, and the brains were cut into 3mm sections and stained with tetrazolium chloride. Negatives were then prepared and analysed for infarct and whole brain area using a Quantimet. An estimate of volume was then calculated from area. Statistical comparions between vehicle (saline) treated and compound treated rats were made using an unpaired, two-tailed Students t-test. Values for absolute volume and percent of total brain area respectively were compared to the control (vehicle) value, in each case.

Results:

In the vehicle-treated group of rats, the total infarcted volume was found to be approximately 154mm^3, constituting 10.4% of total brain volume. No evidence of infarcted tissue observed in any of the sham-operated rats. Dextromethorphan, carbetapentane and compound 11 reduced the area of ischaemic damage produced by occlusion of the middle cerebral artery in the rat (Table 1 and Fig. 1). An estimate of the potency of each compound was obtained by fitting the available data to a sigmoidal dose response curve. These results are shown in Table 2 and Fig.2.

Compound 11 was the most potent compound in protecting against ischaemic damage in this model, with an approximate ED_{50} of 5.3mg/kg. This compound was 13-fold more potent than DM and 5-fold more potent than the parent compound CBP. Compounds B and D are less potent, and did not produce 50% inhibition even at the highest doses tested (60 and 50mg/kg respectively). Although there does not appear to be a good relationship between neuroprotective potency and binding affinities (DM and compound D are the most potent against [^{3}H]DM), there is a very good *in vivo* correlation between neuroprotective and anticonvulsant potency (r=0.965). Possible explanations for the lack of correlation between *in vitro* and *in vivo* potency include differences in distribution and metabolism of these

compounds; or the existence of DM receptor subtypes and an unknown role of these in the anticonvulsant and neuroprotective effects of DM. Another possible explanation for the lack of effect of D in spite of its high binding affinity is that this compound acts as an antagonist or partial agonist at DM receptors. D at a concentration which has only slight effects on infarct size appeared to reduce the effect of the lowest dose of 11 tested. However, this effect was small and not significant. An investigation to see whether increasing the dose of D or decreasing the dose of 11 would increase this apparent antagonistic effect was not possible due to lack of compound.

Table 1: Reduction in infarct volume by DM and carbetapentane analogues in rat MCA-O (expressed as absolute values and as percent of total brain area)

Compound	Dose (mg/kg)	Infarct Volume (mm^3)	% brain volume	n
Vehicle	-	153.89 ± 11.99	10.42 ± 0.76	7
DM	15	140.95 ± 20.58	9.40 ± 1.24	5
	30	109.18 ± 12.71*	7.09 ± 0.60**	5
	60	92.42 ± 9.30**	5.91 ± 0.74**	5
CBP	15	116.80 ± 41.36	7.69 ± 2.63	5
	30	78.62 ± 13.84**	4.90 ± 0.84***	5
	60	36.53 ± 14.13***	2.36 ± 0.91***	5
11	7.5	53.77 ± 16.66***	4.01 ± 1.22***	5
	15	44.61 ± 13.82***	3.30 ± 0.99***	5
	30	13.88 ± 6.80***	0.93 ± 0.45***	5
B	60	120.24 ± 7.97	8.06 ± 0.56*	5
D	15	145.41 ± 26.34	9.91 ± 1.91	5
	50	113.14 ± 25.67	7.95 ± 1.70	5
D + 11	50 + 7.5	78.40 ± 7.70*** [a]	5.31 ± 0.61*** [a]	5

* $p < 0.05$
** $p < 0.01$
*** $p < 0.01$.

[a] not significantly different from 11 7.5mg/kg alone

Table 2: Approximate ED_{50} values for DM and carbetapentane analogues in rat MCA-O

Compound	ED_{50} (mg/kg)	slope	r
DM	70.42	-1.18	0.918
CBP	28.09	-1.63	1.000
11	5.30	-1.05	0.845
B	>60	-	-
D	>50	-	-

Fig 1.

Reduction in Infarct Volume by Dextromethorphan and Carbetapentane Analogues in Rat MCA-O

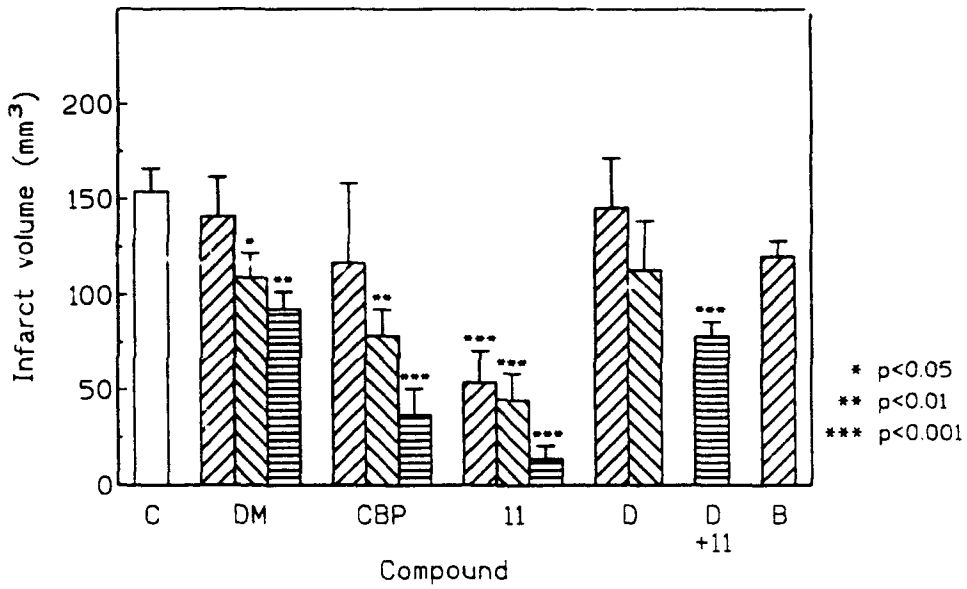
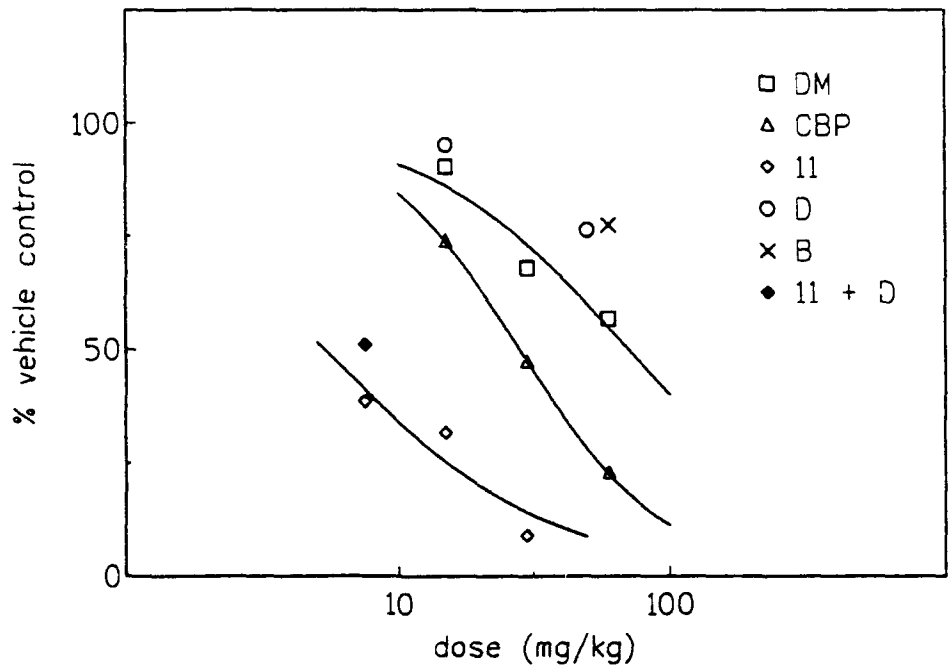


Fig 2

Relative Potencies of DM and Carbetapentane Analogues in Rat MCA-O



Report (June 1992 - January 1993)

K. Bevan
& N.G. Bowery

A comparison of the binding characteristics of [^{3}H]PK-11195, [^{3}H]dextromethorphan, and [^{3}H]N,N-di(o-tolyl)guanidine (DTG) in ischaemic (middle cerebral artery-occluded) and normal (sham) tissue in rat brain has been completed. The binding density of PK-11195, a marker for neuronal damage, was significantly increased in ischaemic tissue as compared with normal tissue, as shown by the increase in B_{max} values obtained in the ipsilateral side of the MCA-occluded rat brain as compared with the contralateral side and with sham-operated rats (table 1). This was accompanied by an increase in the affinity (K_d) of the binding sites. However, there is no evidence of any differences in either K_d or B_{max} values between ischaemic and normal tissue for DM and DTG binding. Although this may just reflect the fact that no changes actually occur under these conditions, other explanations should be considered:

(1) The brains were tested as a whole homogenate, and thus this method would probably not detect changes in discrete regions of the brain. In order to investigate this, an autoradiographic study is being carried out in rat brain sections. To date the assay methods have been established, and experiments performed to investigate the distribution of [^{3}H]DM in normal (untreated) rat brain. These autoradiograms are currently awaiting Quantimet analysis. MCA-O and sham-operated rat brains have been prepared and are awaiting binding experiments for autoradiography.

(2) DM appears to bind to more than one site in rat brain (unlike either PK11195 or DTG), and it is possible that only one of these sites is modified in infarcted tissue. So that each site may be compared separately, an attempt is being made to resolve the data into two sites. However, this analysis is made difficult by the limited number of data points to each curve, made necessary by experimental restraints.

(3) The experiments have used 4-day MCA-O rats; it is possible that changes in DM or DTG binding characteristics follow a different time course, reaching a maximum after a much shorter or much longer period of time. This will not be investigated in conventional binding experiments, but if the autoradiographic studies indicate differences in regional DM binding sites, experiments to determine the time course of these changes may be performed.

In addition to the work on the rat MCA-O model described above, the carbetapentane analogues have been evaluated in rat and guinea-pig brain against [^{3}H]DM and in rat brain against [^{3}H]TCP. These results are to be published as a BPS oral communication in April. The abstract for this is appended.

Table 1. K_d and B_{max} values (in nM and pmol/mg, respectively) for PK-11195, DM and DTG binding in MCA-O and sham-operated rats.

GROUP	MCA				SHAM			
	ipsilateral		contralateral		ipsilateral		contralateral	
	K_d	B_{max}	K_d	B_{max}	K_d	B_{max}	K_d	B_{max}
PK-11195	8.31* ±3.40	3.49* ±1.37	1.93 ±0.28	0.57 ±0.07	2.15 ±0.64	0.52 ±0.05	1.98 ±0.11	1.00 ±0.39
DM	266.1 ±51.8	3.71 ±0.43	369.1 ±57.3	3.96 ±0.57	334.4 ±48.5	3.91 ±0.26	460.9 ±118.3	4.66 ±0.89
DTG	76.62 ±5.57	3.37 ±0.41	65.88 ±8.96	3.55 ±0.46	72.13 ±10.4	4.01 ±0.22	73.34 ±8.43	4.08 ±0.44

* $p < 0.05$

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22-1-93

Carbetapentane analogues: anticonvulsant agents acting via dextromethorphan receptors?

K.A. Bevan, [1]A.H. Newman, [1]S.N. Calderon, [2]F.C. Tortella and N.G. Bowery, Dept. Pharmacology, School of Pharmacy, London WC1N 1AX, [1]N.I.D.A.-Addiction Research Centre, Baltimore, M.D. 21224 and [2]W.R.A.I.R., Washington D.C. 20307-5100.

It is unclear whether the anticonvulsant activity of the antitussive agent dextromethorphan (DM) is mediated via DM binding sites in rat brain or via the NMDA receptor associated phencyclidine (PCP) site, to which DM also binds (Newman *et al.*, 1992). Carbapentane (2-[2-(diethylamino)-ethoxy] ethyl-1-phenyl-1-cyclopentylcarboxylate, CBP) which is another antitussive and anticonvulsant is also a ligand for the DM site (Tortella & Mussachio, 1986). In an attempt to determine the involvement of DM, as compared to PCP, sites in the action of CBP a series of analogues were synthesised (Calderon *et al.*, 1991) and their affinities for the DM and PCP sites determined. Within the CBP molecule, two sites were modified (Table): the cyclopentyl ring was expanded (n) and/or the ester function was replaced (X). The activity of these compounds as anticonvulsants was assessed using the rat supramaximal electroshock (MES) test and compared with binding activities at the DM and PCP binding sites in rat brain, using [^{3}H]DM and [^{3}H]thienylcyclohexyl-piperidine ([^{3}H]TCP) respectively (Newman *et al.*, 1992).

Protection against MES-induced seizures was observed with CBP and three of the analogues (Table), with one of these (2) being more potent than the parent compound. All of the CBP analogues inhibited [^{3}H]DM binding in rat brain. Although there appears to be only a weak correlation between anticonvulsant activity and affinity for the DM site, there may still be a functional relationship between binding and *in vivo* activity. The compounds appear to bind to more than one site (low slope factors), and the significance of the high and low affinity sites in the anticonvulsant effect of these compounds is not known. Importantly, however, none of the compounds (up to 10μM) showed any activity at the PCP site in rat brain and thus it seems unlikely that the anticonvulsant effect is mediated *via* the NMDA receptor. Neither CBP nor compound 2 produced any behavioural toxicity at 100 mgkg^{-1}. In addition, compound 2 had no pro-convulsant activity at doses up to 7 times its MES ED_{50}. This protective index exceeds those of currently available anticonvulsant agents, and could have exciting implications for future anticonvulsant therapy.

Compound	n	X	[^{3}H]DM K_i (μM ± s.e.m.)	slope	MES ED_{50} (μmolkg^{-1}, 95% CL)
1(CBP)	1	$-CO_2$	2.8±0.92	-0.37	48 (31-72)
2	1	$-CH_2O-$	3.2±0.9	-0.33	16 (9-31)
3	2	$-CH_2O-$	0.6±0.2	-0.25	86 (50-151)
4	1	-CONH-	>10	---	>50mgkg^{-1}
5	2	$-CO_2-$	0.5±0.2	-0.29	173 (80-375)
6	1	$-CH_2NH-$	4.2±1.2	-0.42	>50mgkg^{-1}
7	1	$-CH_2N(CH_3)-$	5.0±1.8	-0.31	>50mgkg^{-1}

(CL = Confidence Limits)

Calderon S.N., Newman A.H., Tortella F.C. (1991) J. Med. Chem. 34 3159-3164
Newman A.H., Bevan K.A., Bowery N.G., Tortella F.C. (1992) J. Med. Chem. 35 4135-4142
Tortella F.C., Musacchio J.M. (1986) Brain Res. 383 314-318

The numbers in the abstract submitted to the British Pharmacological Society Meeting (April 1993) correspond to the number in Tables 1-4 (carbetapentane) as follows:

BPS abstract key	Corresponds to
2	11
3	12
4	21
5	23
6	24
7	26

June 1992 - August 1992

BINDING OF CARBETAPENTANE ANALOGUES IN RAT AND GUINEA PIG BRAIN

The binding of a series of carbetapentane (CBP) analogues at the dextromethorphan binding site in rat and guinea pig brain, and the NMDA receptor-associated PCP site in rat brain, was assessed using [^{3}H]dextromethorphan ([^{3}H]DM) and [^{3}H]thienylcyclohexylpiperidine ([^{3}H]TCP) respectively. The methods used are described below. All compounds were dissolved in distilled water at 10^{-2}M, and diluted with Tris-HCl as described in the binding assay methods. They were then diluted and dispensed into 1ml miniblocks using a TECAN, in a final volume of 0.5ml, and assayed at concentrations from 1nM to 10μM. Filtering was performed using a Brandel M-48R cell harvester, and samples were counted in 5ml of 'Ecoscint A' scintillation fluid after an extraction period of at least 12 hours, using a Beckman liquid scintillation counter.

[^{3}H]DM Binding.

Membrane Preparation: Frozen rat and guinea pig brains (Charles River) were thawed, suspended in 10 volumes of ice-cold 0.32M sucrose, and homogenised using a Polytron (30s, setting 6). This was centrifuged at 1000g for 20 minutes, the pellet discarded and the supernatant centrifuged at 100,000g for 60 minutes. The pellet was then resuspended in 3 volumes of 50mM Tris-HCl (pH 7.4 at 23°C) and frozen at -80°C.

Binding Assay: 50μl of [^{3}H]DM (NEN, 85.9Ci/mmole) at a concentration of 5nM was incubated for 20 minutes at 23°C, with 400μl of either rat or guinea pig brain homogenates (final concentration approximately 200μg/ml), and 50μl of either buffer (for total binding), 100μM cold DM (for non-specific binding) or cold compound at the appropriate concentration. The reaction was terminated by dilution with 0.5ml of ice-cold wash buffer (Tris-HCl containing 100mM choline chloride and 0.01% Triton X-100), followed by rapid filtration through GF/B filters presoaked at least two hours in wash buffer (to reduce non-specific binding). The filters were washed with 4x1ml wash buffer and counted as described above.

[^{3}H]TCP Binding.

Membrane preparation: Frozen rat brains (minus cerebellum) were thawed and homogenised in 10 volumes of ice-cold 0.32M sucrose (30s, Polytron setting 6). The homogenate was then centrifuged at 1000g for 20 minutes, the pellet discarded, and the supernatant centrifuged at 20,000g for 20 minutes. The pellet from this was resuspended in 10 volumes of ice-cold distilled water, vortexed, and allowed to stand for 10 minutes before being centrifuged at 8000g for 20 minutes. The supernatant was then used to wash the buffy uppercoat away from the mitochondria-enriched pellet and centrifuged for 20 minutes at 40,000g to give a crude synaptic plasma membrane pellet. This was washed twice in distilled water at 48,000g for 20 minutes, and the resulting pellet frozen at -20°C for at least 18 hours. The pellets were then thawed, resuspended in 5mM Tris-HCl (pH 7.7 at 0°C), vortexed, and washed a total of 6 times at 48,000g. The resulting pellet was resuspended in 3 volumes of 5mM Tris-HCl (pH 7.7 at 23°C), and frozen at -80°C.

Binding Assay: 50μl of [^{3}H]TCP (NEN, 40.8Ci/mmole, final concentration 5nM) was incubated for 60 minutes at 23°C with 400μl of rat brain crude synaptic plasma membranes (final concentration approximately 100μg/ml), and 50μl of either buffer (for total binding), 10μM phencyclidine (PCP, for non-specific binding) or cold compound at the appropriate concentration. The reaction was terminated by dilution with ice-cold wash buffer (5mM Tris-HCl pH 7.7 at 0°C), followed by rapid filtration through GF/B filters presoaked at least 2 hours in 0.5% polyethyleneimine. The filters were then

washed 4 times with 1ml wash buffer and counted as before.

Results

Results were expressed as IC_{50}'s (concentration of compound which produces 50% inhibition of total specific binding), using "GraphPad" sigmoidal curve fitting program. K_i values were then calculated using the equation $K_i = IC_{50}/[1+(L/K_d)]$ where L is the concentration and K_d the affinity constant of the ligand.

Dextromethorphan was active in both guinea-pig and rat brain, with K_i values of 63.7nM and 501.7nM respectively. All of the carbetapentane analogues showed potent activity against [^{3}H]DM in guinea-pig brain, with K_i's ranging from 1 to 300nM (Table 1). The analogues were less active in rat brain, with K_i's in the micromolar range except for compounds 23 and D (495nM and 252nM respectively, Table 2). None of the analogues showed any significant activity against the [^{3}H]TCP binding site in rat brain. The standard PCP was active (IC_{50} 58.6±14.6nM, slope -0.79±0.04), but the maximum activity showed by any of the analogues was compound 24 which inhibited by 13% at 10μM (Table 3).

The slopes obtained with these compounds were all significantly less than 1, suggesting binding to more than one site. Indeed, fitting the data to a two-site competition curve gave a significantly better correlation than to a one-site competition curve (these models assume a slope of 1). Table 4 shows the values obtained when the mean inhibition values for each concentration of compound were fitted to a two-site competition model. In each case there appears to be a high affinity and a low affinity site, in varying proportions. In general, the IC_{50} obtained from the one-site fit is closest to that for the site which is present in larger amounts.

Table 1: Inhibition of [^{3}H]DM Binding in Guinea-Pig Brain by Carbetapentane Analogues.

Comp[a]	IC_{50} (nM)	slope	K_i (nM)	n
DM	66.8±25.7	-0.58±0.07	63.7±24.4	5
CBP	15.1±9.3	-0.35±0.04	14.4±8.8	5
11	1.51±0.71	-0.23±0.03	1.43±0.67	5
12	4.43±1.90	-0.41±0.07	4.24±1.82	5
21	313.7±61.6	-0.59±0.10	299.7±59.1	5
23	1.63±0.78	-0.28±0.02	1.56±0.74	4
24	49.4±28.4	-0.24±0.02	47.1±27.1	5
26	40.7±24.0	-0.33±0.06	38.9±23.0	5
A	125.3±36.4	-0.56±0.04	119.5±34.6	5
B	102.7±38.6	-0.49±0.11	97.8±36.7	5
C	59.9±38.9	-0.44±0.09	57.2±37.2	5
D	1.31±0.32	-0.27±0.03	1.08±0.30	5

Table 2: Inhibition of [^{3}H]DM Binding in Rat Brain by Carbetapentane Analogues.

Comp[a]	IC_{50} (nM)	slope	K_i (nM)	n
DM	513.7±165.0	-0.61±0.06	501.6±160.9	5
CBP	2848.8±364.4	-0.37±0.09	2783.2±844.3	5
11	3194.8±934.7	-0.33±0.07	3118.4±910.5	5
12	574.0±152.2	-0.25±0.05	561.0±148.9	5
21	15377.1±8661.1	-0.53±0.12	15054.1±8497.6	5
23	507.0±157.4	-0.29±0.09	495.1±153.9	4
24	4154.1±1205.5	-0.42±0.12	4059.4±1175.8	5
26	5010.7±1845.1	-0.31±0.03	4904.2±1811.5	5
A	3590.9±1000.5	-0.45±0.08	3507.2±975.5	5
B	6179.0±1867.6	-0.45±0.10	6042.2±1831.1	5
C	1681.7±792.4	-0.41±0.10	1645.6±777.3	5
D	258.7±145.0	-0.32±0.07	252.4±141.5	4

Table 3: Effect of Carbetapentane Analogues on [^{3}H]TCP Binding in Rat Brain.

Comp[a]	% Inhibition @ 10μM	n
DM	N/T	-
CBP	10.2±4.1	5
11	11.9±2.2	5
12	8.1±1.8	5
21	7.1±2.4	5
23	8.9±1.2	5
24	13.1±1.8	5
26	11.2±2.5	5
A	4.6±3.4	5
B	5.1±3.9	5
C	3.0±2.5	5
D	5.7±2.0	5

N/T = not tested

Table 4(a): Inhibition of [^{3}H]DM Binding in Giunea-Pig Brain: 2-Site Curve Fit.

Comp[d]	IC_{50} 1 (%)	IC_{50} 2 (%)	r
DM	5.11 (45.7)	394.3 (54.3)	0.996
CBP	0.59 (55.5)	375.8 (44.5)	0.999
11	0.20 (67.5)	1247 (32.5)	0.898
12	1.05 (63.6)	956.8 (36.4)	0.979
21	2.20 (26.1)	530.4 (73.9)	0.979
23	0.39 (72.2)	942.8 (27.8)	0.903
24	0.10 (46.8)	1309 (53.2)	0.974
26	0.23 (44.3)	459.9 (55.7)	0.925
A	16.8 (55.5)	1136 (44.5)	0.993
B	0.64 (32.0)	364.2 (68.0)	0.978
C	0.73 (44.1)	349.5 (55.7)	0.974
D	0.18 (63.5)	365.9 (36.5)	0.975

Table 4(b): Inhibition of [^{3}H]DM Binding in Rat Brain: 2-Site Curve Fit.

Comp[d]	IC_{50} 1 (%)	IC_{50} 2 (%)	r
DM	104.1 (69.7)	9156 (30.3)	0.999
CBP	78.5 (52.0)	11470 (48.0)	0.999
11	8.14 (31.4)	12620 (68.6)	0.879
12	5.62 (35.1)	15720 (64.9)	0.951
21	197.4 (55.1)	18850 (44.9)	0.993
23	0.92 (39.0)	7029 (61.0)	0.894
24	1.25 (18.7)	6930 (81.3)	0.962
26	1.84 (25.2)	10640 (74.8)	0.920
A	65.5 (36.8)	15590 (63.2)	0.996
B	170.0 (44.2)	47380 (55.8)	0.997
C	- -	2708 (81.4)	0.901
D	0.03 (32.0)	1894 (68.0)	0.968